0013-7227/83/1135-1629$02.00/0
Endocrinology

Vol. 113, No. 5
Printed in U.S.A.

Effects of Estradiol Treatment on Rabbit Luteal Adenylyl Cyclase: Loss of Luteinizing Hormone Receptors and Attenuation of the Regulatory *N* Component Activity*

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ABSTRACT. We have reported previously that exogenously administered estradiol (E_2) results in attenuation of the LH response of rabbit luteal adenylyl cyclase (~50% less activity than control). This was accompanied by a much lesser reduction in the response of the system to the β-adrenergic agonist isoproterenol (~35% less activity than control). The purpose of the study reported here was to determine if the decreased responsiveness of adenylyl cyclase was the result of altered hormone receptor levels. To this end, hormone receptors were assessed by Scatchard analysis of specific binding. We confirmed that 4-day E_2 treatments, which elevated serum E_2 levels from 5 to 21 pg/ml, resulted in decreases in LH- and isoproterenol-stimulated adenylyl cyclase activities by 52% and 20%, respectively. In addition, we found that NaF-stimulated activity was also decreased by 20%. Basal adenylyl cyclase activity was unaffected. Upon assessment of the LH and β-adrenergic receptor levels in luteal membranes, we found that E_2 treatment resulted in marked reduction in LH receptor levels to 28% of the control value without changes in the levels of β-adrenergic receptors. In view of the concomitant changes in the responsiveness of luteal membranes to isoproterenol and NaF, we determined whether E_2 treatment affected luteal membrane levels of the stimulatory nucleotide-binding regulatory component (*N*) of adenylyl cyclase. *N* component activity was measured using a reconstitution assay that employs the stimulatory *N* component-deficient *cyc*⁻ variant of the S49 mouse lymphoma cell line as an acceptor for luteal *N* component. Using this assay, we found that luteal membrane *N* component activity was reduced by 20–25% in E_2-treated rabbits compared to that in control rabbits. All of the changes noted above were statistically significant.

The results uncovered two heretofore unrecognized effects of E_2 treatment: 1) loss of LH receptors, and 2) modification of the membrane component responsible for coupling of stimulatory receptors to the catalytic component of adenylyl cyclase. (*Endocrinology* **113:** 1629, 1983)

THE FACT that estradiol (E_2) is the primary luteotropin in the rabbit is well established (1–6). Yet, we found that supraphysiological levels of E_2, administered by either injection (7) or Silastic implant (8), caused a decrease in luteal LH-responsive adenylyl cyclase activity. In the latter study, the response of the adenylyl cyclase system to the β-adrenergic agonist isoproterenol (Iso) was attenuated as well, although to a much lesser extent than that to the gonadotropin LH.

Exogenous E_2 could be affecting any or all of the three major components of the adenylyl cyclase system: the hormone receptor, the catalytic unit, or the nucleotide-binding regulatory (*N*) component. In addition to being the site of action of Mg (9, 10), guanine nucleotides (9, 11, 12), NaF (13, 14), and cholera toxin (15), the *N* component is the entity that couples hormone receptors to the catalytic unit of the adenylyl cyclase system (13, 16–19).

Recently, measurement of *N* component function has become feasible using reconstitution assays that employ the so-called *cyc*⁻ variant of the S49 mouse lymphoma cell. These cells contain membrane-bound β-adrenergic receptors and the catalytic unit of the adenylyl cyclase system. However, they are deficient in a functional stimulatory regulatory *N* component (20). Membranes from this variant can act, therefore, as recipients for *N* components from other tissue sources.[1] Thus, *cyc*⁻ adenylyl cyclase activity can be reconstituted to a level dependent upon the quantity and quality of the exogenously added *N* component. Reconstitution assays of this kind have been used to measure *N* component activities in a variety

Received January 17, 1983.
* This work was supported by NIH Grants HD-09581 (to L.B.) and HD-05823 (to H.J.K.). Presented in part at the 64th Annual Meeting of The Endocrine Society.
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[1] It is now known that there are two separate regulatory *N* components: one that mediates stimulatory effects of hormones such as LH and epinephrine acting through β-adrenergic receptors and often referred to as N_s, and another that mediates inhibitory effects of hormones such as opioids and epinephrine acting through α_2-adrenergic receptors and often abbreviated as N_i. The *cyc*⁻ cell line lacks N_s but contains active N_i, and reconstitution assays using *cyc*⁻ membranes measure N_s activity. Throughout this article, we will assay for N_s only, but, for the sake of simplicity, will speak of and refer to it simply as *N*.

of tissues (10, 13, 21, 22). To evaluate the effect of E_2 treatment on the luteal hormone-responsive adenylyl cyclase system, we developed a quantitative assay for luteal *N* component activity. Using this assay as well as specific binding assays for LH and β-adrenergic receptors, the effects of E_2 administration were localized to both the level of LH receptors and the level of the regulatory *N* component.

Materials and Methods

Materials

Inorganic ^{32}P was purchased from CintiChem, Inc. (Tuxedo, NY), ^{125}I (carrier-free) was obtained from Iso-Tex Diagnostics (Friendswood, TX), and [^{3}H]cAMP was purchased from Schwarz/Mann (Orangeburg, NY). Xylazine (2.5 mg/kg BW; Rompun, Cutter Laboratories, Inc., Shawnee, KS) and ketamine (15 mg/kg BW; Ketaset, Bristol Laboratories, Syracuse, NY) were used as anesthetics. hCG for injection was obtained from Ayerst Laboratories (New York, NY) and was dissolved in 0.15 M saline to yield a concentration of 250 IU/ml. Highly purified hCG for iodination (hCG CR119) and LH (NIH LH-B9) were obtained from the NIH. The LH was kept as a stock solution of 1 mg/ml in water. (−)Iso was a gift from the Sterling Winthrop Research Institute (Rensselaer, NY) and was kept as a 10-mM stock solution in 1 mM HCl. NaFl (Fisher Scientific Co., Waltham, MA) was kept as a 1-M stock solution. (−)Propranolol was a gift from Ayerst, and hydroxybenzylpindolol (HYP) was a gift from Sandoz (East Hanover, NJ). Progesterone and E_2 derivatives for iodination were steroid-11-hemisuccinate-tyrosine-methyl-esters (steroid-11-TME) and were gifts of Dr. John E. Pike (Upjohn Co., Kalamazoo, MI). Creatine phosphate and creatine kinase were obtained from Calbiochem (La Jolla, CA); myokinase, ATP (Tris-salt), GTP, cAMP, EDTA, and Tris were purchased from Sigma Chemical Co. (St. Louis, MO); [α-^{32}P]ATP (200–500 Ci/mmol), synthesized according to the procedure of Walseth and Johnson (23) and purified according to the method of Birnbaumer *et al.* (24), was supplied by the Core Laboratory for Cyclic Nucleotide Research, Center for Population Research and Studies on Reproductive Biology, Baylor College of Medicine (Houston, TX).

E_2-filled Silastic capsules (id, 3.35 mm; od, 4.65 mm; length, 12 mm) were prepared according to the method of Holt *et al.* (25). [^{125}I]Iodo-HYP ([^{125}I]IHYP) was prepared by iodination of HYP according to the method of Maguire *et al.* (26) and was purified by high pressure liquid chromatography, as described by Bearer *et al.* (27). [^{125}I]Iodo-hCG was prepared using the lactoperoxidase procedure developed for LH (28) as modified for hCG (29).

Animals

Virgin New Zealand White rabbits (3–4 kg) were housed in individual cages in air-conditioned quarters and were fed Purina rabbit chow *ad libitum* for at least 15 days before the initiation of experiments. Pseudopregnancy was induced by the iv injection of 75 IU hCG. The day after hCG injection was designated day 1 of pseudopregnancy.

Treatments and preparation of corpora luteal (CL) membranes

Rabbits were treated with E_2 for 4 days, starting on the morning of day 5 of pseudopregnancy and ending on the morning of day 9 of pseudopregnancy. Treatments were initiated by implanting two E_2-filled Silastic capsules (see *Materials*) sc under light anesthesia (see *Materials*) and were terminated by killing the animals by cervical dislocation. Control rabbits were treated identically, except that instead of E_2-filled capsules, empty Silastic capsules were implanted. Ovaries were removed and placed in ice-cold Krebs-Ringer bicarbonate buffer prepared with half the recommended amount of $CaCl_2$ (30).

CL were dissected free of the ovaries, cleaned of adhering interstitial tissue, and kept in ice-cold Krebs-Ringer bicarbonate buffer until further processing (30 min to 1 h). Homogenization and membrane particle preparation were performed as described previously (31). The experiment was performed twice. The first time, all of the CL from a group (five rabbits per group) were pooled and were used to supply examples of representative assay data. The second time, the CL from each animal were processed separately so that membrane particle preparations from individual rabbits were obtained and group means ± SEMs could be calculated.

Adenylyl cyclase assays

Adenylyl cyclase activity in 10-μl aliquots of membrane preparations was determined, as described previously (24), at 32.5 C for 10 min in medium containing 3.0 mM ATP (with 5 × 10^6 cpm [α-^{32}P]ATP), 5.0 mM $MgCl_2$, 1 mM EDTA, 1.0 mM cAMP (containing 10,000 cpm [^{3}H]cAMP), 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, 0.02 mg/ml myokinase, and 25 mM Tris-HCl. Unless otherwise noted, the concentrations of LH, Iso, and NaF were 10 μg/ml, 100 μM, and 10 mM, respectively. GTP was present at a concentration of 95 μM in all tubes other than those containing NaF. The [^{32}P]cAMP formed was quantitated by the method of Salomon *et al.* (32), as modified by Bockaert *et al.* (33). Results are expressed as picomoles of cAMP formed per min/mg protein. Protein was determined by the method of Lowry *et al.* (34) using BSA (fraction V, Armour Pharmaceutical Co., Chicago, IL) as standard.

Preparation of cyc$^-$ membranes

Cyc$^-$ cells were grown in Dulbecco's Modified Eagle's Basal Medium supplemented with 10% heat-inactivated horse serum in suspension culture, as described by Coffino *et al.* (35). Cells were harvested and prepared for lysing by washing three times with Puck's Saline G (without divalent cations). The resuspended cells (2.5–3 × 10^6 cells/ml) were then lysed, and 43,000 × *g* particles (*cyc*$^-$ membranes) were prepared according to the procedure of Ross *et al.* (36), except that $MgCl_2$ was omitted from all buffers. The *cyc*$^-$ membranes were resuspended at an approximate protein concentration of 10–15 mg/ml and were aliquoted and stored at −70 C in 100-μl fractions.

Extraction of the luteal N component

Luteal membranes (1.0–1.5 mg protein in 200–300 μl) tha had been stored at −70 C were diluted with 2.5 ml 25 mM Tris-

HCl-1 mM EDTA, pH 7.5 (TE), and centrifuged at 100,000 × *g* for 30 min. The supernatants were discarded, and 250 μl 1% cholate in TE were added to the pellets. The pellets were resuspended and mixed for 15 sec every 5 min for 1 h in a cold room. These cholate-membrane mixtures were then centrifuged for 60 min at 100,000 × *g*, and the supernatants (cholate extracts) were transferred to clean test tubes. The supernatants were diluted with 1% cholate in TE to three different concentrations ranging from 50–300 μg/ml. To the pellets were added 125 μl 1% cholate in TE, and the pellets were resuspended. To selectively inactivate the adenylyl cyclase catalytic unit, the cholate extracts and resuspended pellets were incubated at 32.5 C for 5 min and at room temperature for 40 min.

Reconstitution assays for luteal N component activity

Cyc^- adenylyl cyclase activity in the presence of luteal *N* component was assessed as follows. Mixtures of 120 μl containing 0.5 mM ATP, 0.1 mM GTP, 1.5 mM $MgCl_2$, 2 mM creatine phosphate, 20 μg/ml creatine kinase, 2 μg/ml myokinase, and 25 mM Na-Hepes (pH 8.0) in the absence or presence of cholate extract (1.25–7.5 μg extract protein; 0.2% cholate) and in the absence or presence of cyc^- membranes (5 mg/ml) were incubated for 20 min at 32.5 C and then placed on ice. Aliquots (10 μl) from each of these mixtures were transferred in triplicate to tubes containing 30 μl 33.3 mM $MgCl_2$, 1.67 mM EDTA, 1.67 mM cAMP (with 10,000 cpm [^{3}H]cAMP), 33.3 mM creatine phosphate, 0.33 mg/ml creatine kinase, 0.03 mg/ml myokinase, and 41.7 mM Na-Hepes, pH 8.0, with 156.7 μM GTP, 156.7 μM GTP plus 83.3 μM isoproterenol, or 16.7 mM NaF. These tubes were incubated for 15 min at 32.5 C, at which time 10 μl [α-^{32}P]ATP (~20 × 10^6 cpm; <10 μM) were added to each tube. The incubations were continued for an additional 20 min, at which time the assays were stopped, and [^{32}P]cAMP was isolated, as previously described (25, 26). The final concentrations in the incubations were 0.1 mM ATP, 20 mM $MgCl_2$, 1 mM EDTA, 1 mM cAMP, 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, 0.02 mg/ml myokinase, 25 mM Na-Hepes, 0.04% cholate, and, when present, 0.25–1.5 μg cholate extract protein, 50 μg of cyc^- protein, 94 μM GTP, 50 μM isoproterenol, and 10 mM NaF.

[^{125}I]IHYP binding assays

To assess β-adrenergic receptors, [^{125}I]IHYP binding assays were performed, as described previously (29), using about 25 μg membrane protein in the presence of up to 5 nM [^{125}I]IHYP (1.2 × 10^6 cpm), 25 mM Tris-HCl (pH 7.5), 1.0 mM EDTA, and 1.0% BSA (final volume, 100 μl). Incubations were carried out for 30 min at 32.5 C. Bound labeled ligand was separated from free by a variation of the polyethylene glycol (PEG) precipitation method described recently (37). Briefly, 1.5 ml ice-cold bovine γ-globulin (2 mg/ml) in 100 mM NaCl, followed by 0.5 ml ice-cold 20% PEG (Carbowax 600), were added to the reaction mixtures. The tubes were left on ice for 10 min, centrifuged for 15 min in a Sorvall RC-5 refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, CT) at 3000 rpm using a Sorvall HL-8 rotor, and the supernatants were discarded by aspiration. The precipitates were resuspended by the addition of 1.5 ml 100 mM NaCl and reprecipitated by the further addition of 0.5 ml 20% PEG. After centrifugation and aspiration of the supernatants, the pellets were counted in a γ-counter to assess the amount of [^{125}I]IHYP bound. Nonspecific binding was measured in the presence of 10 μM (−)propranolol. All points were measured in triplicate. The data were subjected to Scatchard analysis to determine the K_d and maximum binding (B_{max}).

[^{125}I]Iodo-hCG binding assays

[^{125}I]Iodo-hCG binding assays were performed, as described previously (29), using approximately 10 μg membrane protein in the presence of up to 5 nM [^{125}I]iodo-hCG (1.2 × 10^6 cpm), 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 1.0% BSA (final volume, 100 μl). The incubations were carried out for 90 min at 32.5 C. The reactions were stopped, and bound ligand was separated from free ligand as described for the [^{125}I]IHYP binding assays. Nonspecific binding was determined in the presence of 10 μg/ml LH. All points were assayed in triplicate, and the data were subjected to Scatchard analysis to determine the K_d and B_{max}.

Preparation of sera and RIAs for E_2, progesterone, and LH

Blood, which was collected by cardiac puncture at the time of death, was allowed to clot at room temperature for about 30 min and was placed in a refrigerator for 4 h, after which it was spun in a refrigerated centrifuge for 10 min to obtain serum.

Serum E_2, progesterone, and LH were assayed by RIAs, as described previously (8). The modifications described by Goodman *et al.* (38) were employed for the E_2 RIA, which used GDN 930 (supplied by Dr. P. Landis Keyes), at a final dilution of 1:1,155,000, as the antiserum. The labeled ligand was [^{125}I]iodo-E_2-11-TME. For the progesterone assays, GDN 337 (supplied by Dr. Gordon D. Niswender) was used as the antiserum at a final dilution of 1:320,000; the labeled ligand was [^{125}I]iodoprogesterone-11-TME. We are indebted to Dr. A. F. Parlow (NIAMDD Pituitary Hormone Distribution Program) for the AFP-8-1-28 antirabbit LH serum and the AFP-559-B rabbit LH used both as the assay standard and the hormone for iodination. The assay was modified such that the antiserum was used at a final assay concentration of 1:8,000,000, and 30,000 cpm labeled LH were added to each tube.

Using these assay procedures, nonspecific binding, percent binding, 50% inhibition, sensitivity, slope of the standard curve, and interassay variation were essentially the same as reported previously (8) with the following exceptions: the 50% inhibition points for the E_2 and LH assays were 4.1 ± 0.2 pg/tube and 2.4 ± 0.1 ng/tube, respectively, and the percent binding of the zero competition tubes for the LH assay was 31.5 ± 1.2%.

Statistics

RIA data were analyzed using a computer program based on the assay statistics described by Midgley, Jr., *et al.* (39) and Duddleson *et al.* (40). The Scatchard analyses employed simple linear regression, since the correlation coefficients were all greater than 0.98. Comparisons between group means were performed using analysis of variance.

Results

RIAs for E_2, progesterone, and LH

To determine the effects of the 4-day E_2 implant on serum hormone concentrations, RIAs for E_2, progesterone, and LH were performed on serum samples derived from blood obtained from the rabbits at the time of death on day 9 of pseudopregnancy. The results of the RIAs are given in Table 1. As can be seen, the E_2 implants caused an approximate 4-fold elevation of serum E_2 and a 36% decrease in serum LH levels relative to the control values. In agreement with previous reports where E_2 was administered either intermittantly (7) or via an implant (8), serum progesterone concentrations were unaffected by the E_2 treatment.

Adenylyl cyclase activities and binding data

To assess the effects of E_2 treatment on the entire intact luteal adenylyl cyclase system, adenylyl cyclase assays were performed using the membranes prepared from individual rabbits. The results of these assays are presented in Fig. 1. E_2 treatment was without effect on basal adenylyl cyclase activity, but affected both hormone- and NaF-stimulated adenylyl cyclase activities. Of these, by far the largest effect of E_2 treatment was on LH-stimulated activity (52% less than control; $P < 0.0005$). The decreases in Iso- and NaF-stimulated activities due to E_2 treatment were only about 20% relative to the control value, but were significant ($P < 0.05$ and $P < 0.01$ for decreases in Iso- and NaF-stimulated activities, respectively).

We pooled the CL from groups of five rabbits each and tested whether the effects of E_2 treatment were associated with alterations of the concentrations required for LH or Iso to elicit half-maximum stimulations (EC_{50}) of adenylyl cyclase activity or with changes in luteal LH or β-adrenergic receptor levels. As illustrated in the *lower panels* of Figs. 2 and 3, the E_2-induced decreases in hormonal responses observed in the experiment shown in Fig. 1 were not due to shifts in the EC_{50} values at

TABLE 1. Effects of E_2 treatment on serum concentrations of E_2, progesterone, and LH

Group	E_2 (pg/ml)	Progesterone (ng/ml)	LH (ng/ml)
Control	5.0 ± 0.9	14.9 ± 2.1	3.9 ± 0.7
E_2-treated	21.0 ± 3.4[a]	15.1 ± 1.8	2.5 ± 0.4[a]

The values given are the mean ± SEM of eight samples.
[a] Significantly different from control by at least $P < 0.05$.

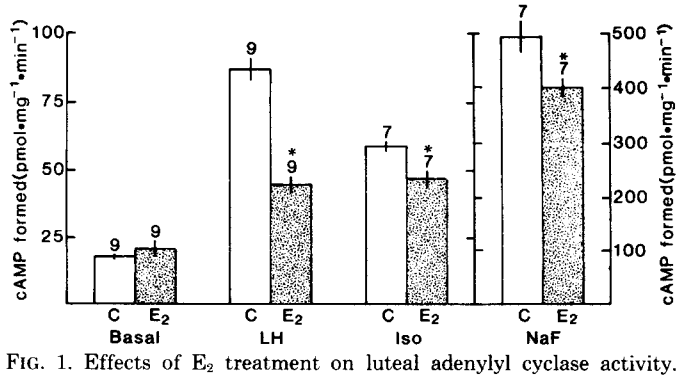

FIG. 1. Effects of E_2 treatment on luteal adenylyl cyclase activity. Each *bar* represents the group mean ± SEM for control (C) or E_2-treated (E_2) rabbits. The *number above each bar* is the number of animals assayed. An *asterisk above a bar* denotes that the mean of the E_2-treated group represented by the bar is significantly different from that of the respective control group by at least $P < 0.05$.

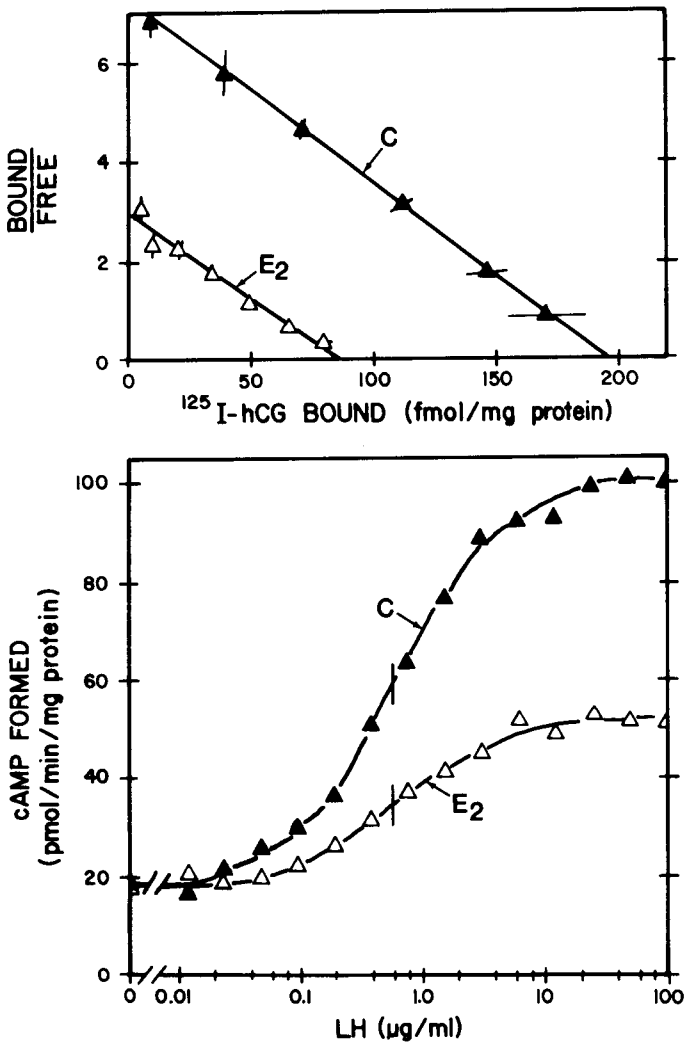

FIG. 2. Effects of E_2 treatment of [^{125}I]iodo-hCG binding (*upper panel*) and adenyl cyclase responses to various concentrations of LH (*lower panel*). The assays were performed on the membrane pools described in the text. ▲, Control (C) group; △, E_2-treated group. The *error bars* in the Scatchard plot are the SEM of triplicate determinations. The K_d for the control group was 26.8 pM, and that for the E_2-treated group was 28.2 pM. The *vertical lines* in the dose-response curve are the EC_{50} values (600 ng/ml for both groups).

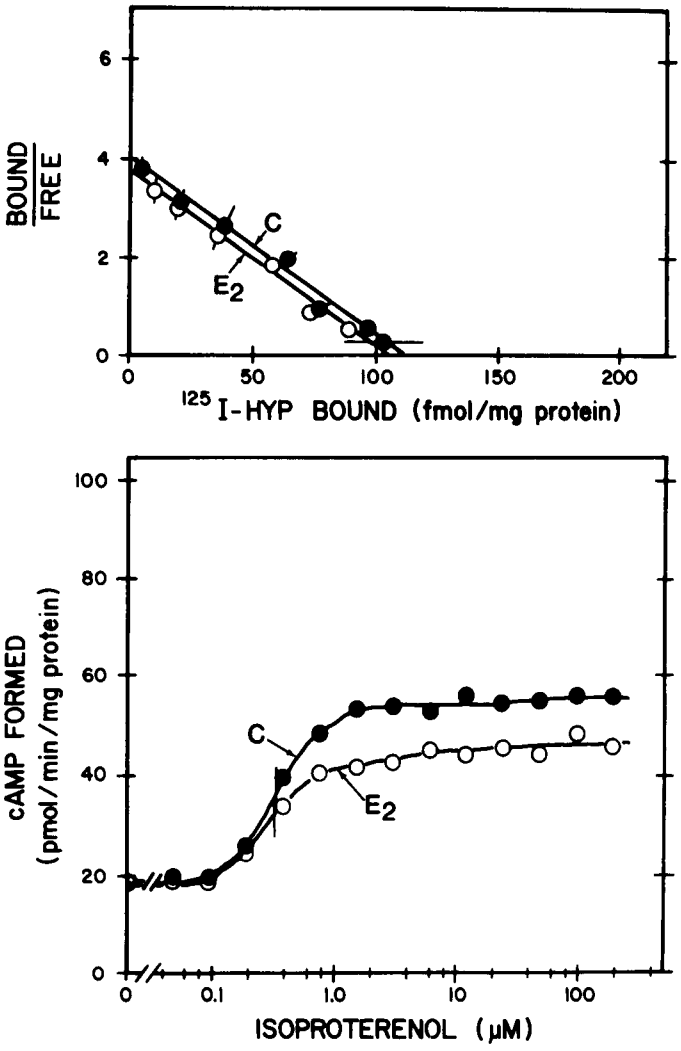

FIG. 3. Effects of E_2 treatment on [^{125}I]IHYP binding (*upper panel*) and adenylyl cyclase response to Iso (*lower panel*). The assays were performed on the membrane pools used in Fig. 2. ●, Control (C) group; ○, E_2-treated group. *Error bars* (*upper panel*) and *vertical bars* (*lower panel*) are described in Fig. 2. The K_d values of binding were 28.0 and 27.6 pM for the control and E_2-treated groups, respectively. The EC_{50} was 575 nM for both groups.

which either LH (Fig. 2) or Iso (Fig. 3) stimulated luteal adenylyl cyclase. Upon testing the levels of LH and β-adrenergic receptors in these membranes, we found that while E_2 treatment resulted in a 56% decrease in LH/hCG receptors (Fig. 2, *top panel*), the level of β-adrenergic receptors appeared unchanged (Fig. 3, *top panel*). In this experiment, the K_d values for [^{125}I]iodo-hCG and [^{125}I]IHYP were 27 and 28 pM, respectively. To confirm these findings, we repeated the experiment using membranes from individual rabbits rather than the membranes pooled from groups of five rabbits.

Figure 4 presents the group means of the B_{max} values obtained for the binding of LH/hCG (Fig. 4, *left panel*) and β-adrenergic (Fig. 4, *right panel*) receptors derived

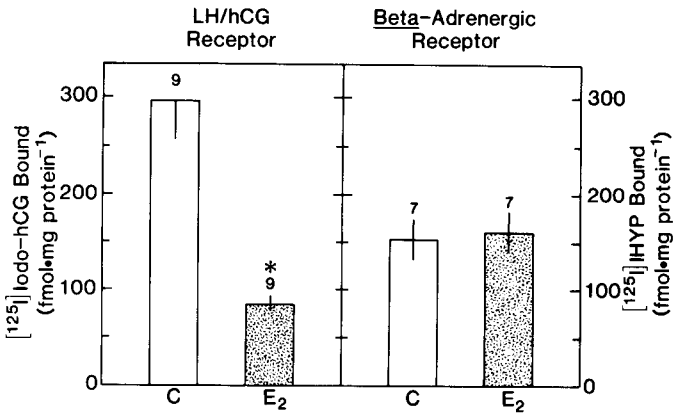

FIG. 4. Effects of E_2 treatment on the number of luteal LH/hCG (*left panel*) and β-adrenergic (*right panel*) binding sites. Each *bar* represents the mean ± SEM of the control group (C) or the E_2-treated group (E_2). The *number above a bar* is the number of animals assayed. An *asterisk above a bar* denotes that the mean of the E_2-treated group represented by the bar is significantly different from that of the respective control group by at least $P < 0.05$.

from Scatchard analyses. The average decrease in luteal LH/hCG-binding sites in the E_2-treated rabbits (72%) was highly significant ($P < 0.005$). In agreement with the results shown in Fig. 3, E_2 treatment was without effect on β-adrenergic binding sites.

The mean ± SEM of the K_d values were as follows: 21 ± 2 and 29 ± 4 pM, respectively, for control and E_2-treated [^{125}I]iodo-hCG binding, and 41 ± 10 and 37 ± 6 pM, respectively, for [^{125}I]HYP binding in luteal membranes of control and E_2-treated rabbits, respectively.

N component activity

The data presented above showed a decrease in both the Iso- and NaF-stimulated activities. We investigated whether these decreases were associated with changes in the activity of the guanine nucleotide- and Mg-binding regulatory component known to be involved in the mediation of the effects of both β-adrenergic receptors and NaF (13, 14, 19). To carry out this measurement, we needed to validate the reconstitution assay described in *Materials and Methods*. As part of this validation procedure, we determined the efficacy of the extraction procedure. To this end, the following experiments were performed. *Cyc*$^-$ reconstituting activity was measured in: 1) the initial cholate-membrane mixture to determine total measurable *N* component activity, 2) the cholate extract obtained from the centrifugation of the cholate-membrane mixture to assess the amount of *N* component activity recovered in the extract, and 3) the residual pellet (resuspended in 1% cholate) to allow for an accounting of total reconstituting activity. As illustrated in Table 2, of the total measurable luteal *N* component activity, 96.3% was accounted for in the cholate extract plus residual pellet, with the cholate extract containing

TABLE 2. Efficiency of cholate extraction of rabbit luteal *N* component activity

Source of *N* component activity	Measured adenylyl cyclase activities			Calculated luteal N activity	
	Total	Intrinsic (no *cyc*⁻)	*Cyc*⁻ only	Absolute	%
Cholate-membrane mixture	46.8 ± 1.5	3.1 ± 0.1	2.8 ± 0.1	40.9 ± 1.5	100.0 ± 3.7
Cholate extract	36.2 ± 0.6	0.9 ± 0.1	2.7 ± 0.1	32.6 ± 0.6	79.7 ± 1.5
Resuspended pellet	10.6 ± 0.2	0.7 ± 0.0	3.1 ± 0.1	6.88 ± 0.2	16.6 ± 0.5
Cholate extract plus resuspended pellet	46.8 ± 0.7	1.6 ± 0.1	5.8 ± 0.1	39.4 ± 0.7	96.3 ± 1.7

Cholate extract is the supernatant of the 100,000 × *g* centrifugation of the cholate-membrane mixture. The pellet of the centrifugation was resuspended in cholate and is termed the resuspended pellet. Except for values in the last column, the values given are expressed as picomoles of cAMP formed in 20 min calculated for 200 μl of the cholate-membrane mixture as starting material. Calculated luteal *N* activity is the total activity minus the intrinsic and *cyc*⁻ activities. Values are the mean ± SD of triplicate determinations.

79.7% of the total measurable activity.

In the experiments comparing the extractability of *N* component activity from luteal membranes of E_2-treated rabbits to that in control rabbits, the ratio [cholate extract/(cholate extract + residual pellet)] × 100 never varied more than 2%, indicating that potential variations in *N* activity due to treatment would not be due to differential yields in extraction of the *N* component.

Finally, we tested whether the assays of *N* activity were linear with respect to the concentrations of luteal cholate extract protein added to the assays. As shown in Fig. 5, where reconstituted *cyc*⁻ adenylyl cyclase activities are plotted against micrograms of luteal cholate extract protein added to the assays, the activities elicited by the extracts increased linearly over a range of cholate extract protein from about 0.25 μg to approximately 3.0 μg.

Using this assay system to test for *N* component activity in the membrane pools from the CL of five control and five E_2-treated rabbits, we found that E_2 treatment caused an approximate 30% decrease in reconstituted adenylyl cyclase activity relative to the control value regardless of whether reconstitution of *cyc*⁻ NaF stimulation (Fig. 5A) or reconstitution of *cyc*⁻ Iso plus GTP stimulation (Fig. 5B) was used to assess *N* component activity.

This measurement was repeated using cholate extracts of membranes prepared from CL of individual rabbits. The group means for these assays are presented in Fig. 6. A 21% decrease was found in *N* activity measured by reconstitution of *cyc*⁻ NaF-stimulated activity (Fig. 6, *left panel*), and a 24% decrease was found in *N* activity measured by reconstitution of *cyc*⁻ Iso-stimulated adenylyl cyclase activity (Fig. 6, *right panel*) when luteal cholate extracts from E_2-treated rabbits were compared to those of control rabbits. These differences were both significant ($P < 0.025$ and $P < 0.005$, respectively).

Discussion

It has been accepted for some time that E_2 is the primary luteotropin in rabbits, yet we believe we have uncovered two heretofore unreported effects of treatment of rabbits with this steroid which may play a role in luteal function: 1) a decrease in LH receptors, and 2) an alteration in *N* component function.

In the present work we have found that supraphysiological concentrations of E_2 results in a decrease in the number of luteal LH/hCG-binding sites. As yet, we do not know the mechanism for this effect of E_2. Since the data reported here result from long term (4-day) E_2 treatment, we cannot be certain that the effects of the treatment are due to direct actions of E_2 on the CL. In view of our finding that E_2 treatment was associated with an approximate 36% decrease in circulating LH, it might be that the loss of LH receptors was the result of a decreased tonic action of this hormone in supporting its own receptors. However, this explanation becomes unlikely in view of the report by Yuh and Keyes (41), who found that LH/hCG binding in hypophysectomized rabbits receiving physiological doses of E_2 is no different from that in control rabbits, suggesting that LH is not necessary for the maintenance of luteal LH receptor. Yet, we cannot rule out pituitary involvement in the phenomenon described here, since the E_2 treatment might result in elevation of serum PRL concentrations, the effect of which on luteal function in rabbits is unknown. Further studies will be needed to uncover the mechanism(s) by which E_2 treatment markedly alters luteal LH receptors.

Regardless of the mechanism by which E_2 treatment results in lowering the number of LH receptors in corpora lutea, the rather close correlation between receptor decrease and decrease in the degree to which the effector system is stimulated is worth noting. It has been reported in the Leydig cell, a LH-responsive steroidogenic tissue, that occupancy of only a small proportion of the receptor sites appears to be necessary for eliciting full responsiveness (42, 43). This finding, which has been labeled the spare receptor phenomenon, is characterized in Leydig cells by a striking leftward shift in the dose-response curves on the effect of hCG to increase cAMP levels (*i.e.* adenylyl cyclase stimulation) with respect to those for

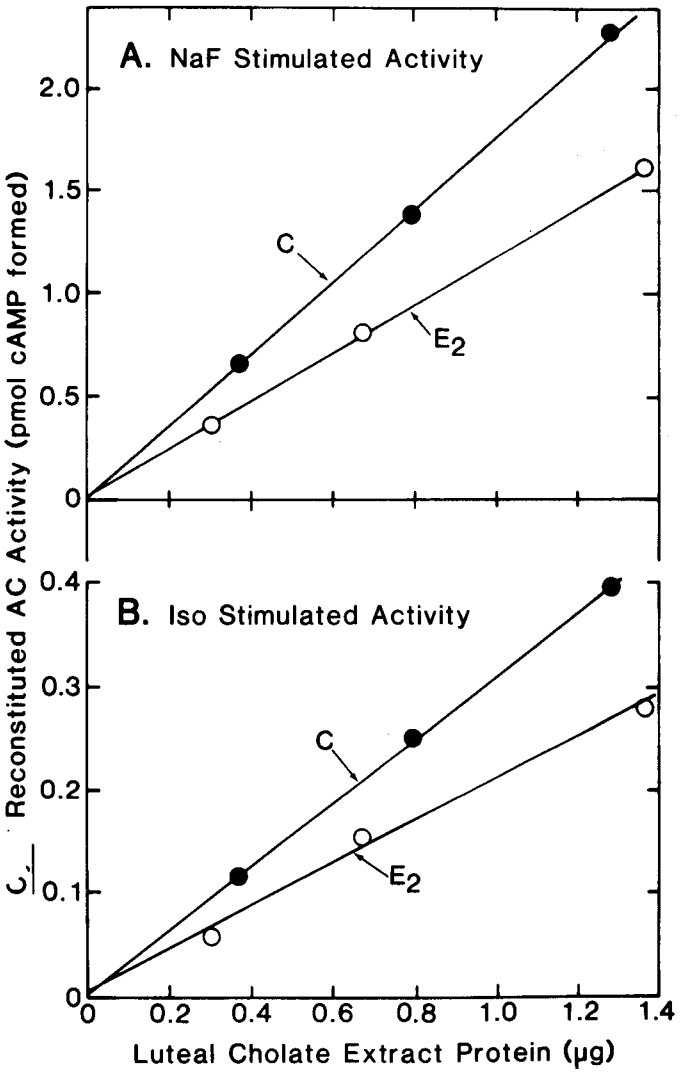

FIG. 5. *Cyc*⁻ reconstituted adenylyl cyclase activities, reflecting luteal *N* component activities in control (C; ●) and E_2-treated (○) rabbits. The data are expressed as picomoles of cAMP formed during a 20-min incubation *vs.* micrograms of cholate extract protein added to the assay. The assays were performed on the same membrane pools as those used for the Scatchard analyses and dose-response curves illustrated in Figs. 2 and 3. Note that all assays were linear throughout the range of cholate extract protein used.

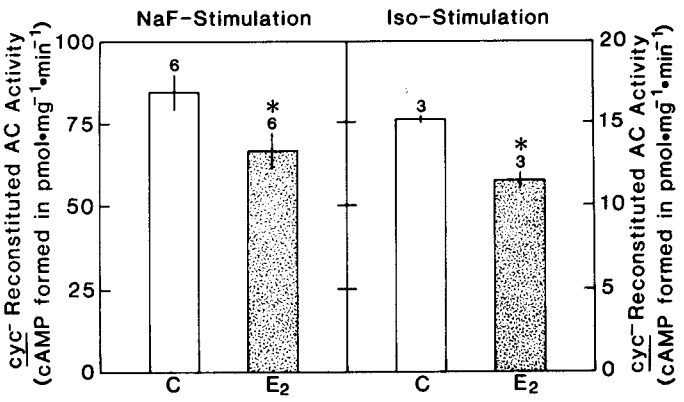

FIG. 6. Effects of E_2 treatment on NaF (*left panel*)- and Iso (*right panel*)-stimulated luteal *N* component activities, as measured by reconstitution assay. Each *bar* represents the mean ± SEM of control (C) or E_2-treated (E_2) rabbits. The *number above a bar* is the number of rabbits assayed. An *asterisk above a bar* denotes that the mean of the E_2-treated group represented by the bar is significantly different from that of the respective control by at least $P < 0.05$.

hCG occupancy of receptors. This suggested a strictly nonlinear coupling event between receptor binding and adenylyl cyclase stimulation in the Leydig cell. Experiments reported here on the effects of E_2, showing quantitatively only minor effects on the activity of the coupling protein *N* (see below) but quantitatively large effects on receptor levels, which correlate with similar changes in hormone responsiveness, strongly suggest that in the CL, LH receptors are limiting and couple to the adenylyl cyclase in a nearly 1:1 ratio, with 50% occupancy eliciting close to 50% stimulation. This is in agreement with a rather close correlation between the K_d for LH binding to rabbit luteal receptors and the EC_{50} with which these receptors stimulate adenylyl cyclase in these membranes (29). It will be of interest to test in the future whether the difference between CL and Leydig cell coupling is due to a difference in the characteristics of the LH receptor or of the coupling mechanism with which the receptors interact with the coupling protein.

Although, as shown in Fig. 4, the attenuated response of the adenylyl cyclase system to LH can be explained by a decrease in the number of LH receptors, the same cannot be said for the comparatively minor but statistically significant decrease in the Iso responsiveness of the system. This finding suggests that E_2 treatment, in addition to causing a decrease in LH receptor number, causes a receptor-independent change in the responsiveness of the adenylyl cyclase system to hormones. In view of the similar change in responsiveness of the system to NaF, the effect of which is known to be dependent on the entity that couples hormone receptors to the catalytic unit, *i.e.* the so-called *N* component, we investigated activity levels of this component. The *N* component of adenylyl cyclase systems is the protein that serves as the interface between hormone receptors and the catalytic unit of the system. Functionally, it is responsible for conferring to the latter Mg-dependent activity and guanine nucleotide sensitivity. The molecular structure of this *N* component has recently been elucidated by Gilman and collaborators (12, 19, 44, 45) to be formed of two subunits: an α-subunit of 42,000–52,000 mol wt which is substrate for the ADP-ribosylating activity of cholera toxin and binds GTP, and a β-subunit of 35,000 mol wt, the function of which is still unknown. Activation of the catalytic component is now recognized to be dependent on activation of the *N* component (11, 14, 44),

a reaction that is dependent on guanine nucleotides and regulated in its rate by Mg ion (9). It appears that hormone receptors exert their effect of stimulate adenylyl cyclases by increasing *N* activation via decreasing the Mg requirement of this reaction (46). Similarly, NaF also exerts its action by increased *N* activation. The central role of the nucleotide-binding *N* component is further emphasized by the fact that it appears to mediate the coupling of not one but several receptors in a single cell. This was suggested by findings made originally in fat cells where up to five hormones were shown to nonadditively stimulate a single adenylyl cyclase system (47). Similarly, the effects of LH and Iso on luteal adenylyl cyclase are nonadditive (48), suggesting that a single system is being affected. Since *N* protein reconstitutes simultaneously NaF, guanine nucleotide, Iso, and prostaglandin stimulation to the *N* component-deficient cyc^- S49 cell membrane adenylyl cyclase (10, 13, 21, 22), it is now recognized that cells contain not only a single type of catalytic unit but also a single type of stimulatory *N* component that is regulated by various receptors in addition to NaF. As applied to the rabbit CL, therefore, the same *N* component and catalytic unit activated by the presence of LH are activated by Iso as well.

The reconstitution assay used by us assessed the function of the luteal membrane *N* component in two ways: 1) assessing the capacity to mediate NaF stimulation, and 2) assessing the capacity to mediate the effect of cyc^- cell β-adrenergic receptors on the catalytic unit of the cyc^- membrane. Our data show that luteal cholate extracts containing *N* component do indeed mediate the effects of both NaF and cyc^- β-adrenergic receptors on cyc^- catalytic component. The data show further that the E_2-induced changes in the degree of reconstitution of NaF-stimulated cyc^- adenylyl cyclase activity are roughly equal to the changes that occur in the degree of reconstitution in cyc^- isoproterenol-stimulated adenylyl cyclase activity. This indicated that E_2 treatment affected luteal *N* component activity either by: 1) decreasing its amount in the membrane, or 2) altering it structurally so as to prevent proper activation under the influence of NaF or hormone receptors.

As noted above, the decreased response of the adenylyl cyclase system to LH can be explained by a loss of LH receptors. However, it is more likely that the loss of LH responsiveness is due to a combination of the decreased number of receptors and the decrease in *N* component activity, but the contribution of the latter is minor compared to that of the former.

Due to the relatively small decrease in *N* component activity (20–25%) which resulted from the E_2 treatment and to the fact that activity was assessed at a single dose and duration of treatment, one might argue that the decreased Iso responsiveness of the system may not have been related to the decrease in *N* component activity. However, both Iso- and NaF-stimulated activities are reduced in CL after E_2 treatment to an extent that is very similar, if not identical, to the extent to which *N* component activity was reduced in these membranes. Thus, it would appear that E_2 treatment of rabbits, such as performed here, results in the loss of *N* component activity and, as a consequence, in a proportional reduction in the degree to which Iso, via the luteal β-adrenergic receptor, stimulates cAMP formation.

Studies are currently under way to determine whether the changes in *N* component activity that occur with E_2 treatment are due to decreased levels of the protein or to an altered structure impeding the proper activation of otherwise normal levels of the protein. One such study involves assessment of ADP ribosylation of the α-subunit of the *N* component and the tests for quantitative and/or qualitative differences between control and E_2-treated membranes.

Finally, if the effect of E_2 on luteal *N* component is a general effect of steroids on other tissues containing functional adenylyl cyclase systems, an entirely new mode of steroid action will have been uncovered. In this sense, our data clearly indicate that adenylyl cyclase activity can be regulated at the level of the nucleotide-binding regulatory component not only as a result of a genetic defect in *N* component, such as that describe by Farfel *et al.* (21) for type I pseudohypoparathyroidism, but by a steroid hormone as well.

Acknowledgments

We are indebted to Dr. R. Iyengar for supplying us with the cyc^- membranes used in this study. We also want to thank Susan Bock for her technical assistance and Suzanne Saltalamacchia for her secretarial work.

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